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DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

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1b. RESTRICTIVE MARKINGS

2a. SECURITY CLASSIFICATION AUTHORITY

DTIC ELECTE DEC 20 1989 S D

3. DISTRIBUTION/AVAILABILITY OF REPORT
Approved for public release; Distribution Unlimited

2b. DECLASSIFICATION/DOWNGRADING SCHEDULE

4. PERFORMING ORGANIZATION REPORT NUMBER(S)

5. MONITORING ORGANIZATION REPORT NUMBER(S)

6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
U.S. Army Medical Research Institute of Infectious Diseases	SGRD-UIS	U.S. Army Medical Research and Development Command

6c. ADDRESS (City, State, and ZIP Code)	7b. ADDRESS (City, State, and ZIP Code)
Fort Detrick, Frederick, MD 21701-5011	Fort Detrick, Frederick, MD 21701-5012

8a. NAME OF FUNDING/SPONSORING ORGANIZATION	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER

8c. ADDRESS (City, State, and ZIP Code)	10. SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)

Clearance, Distribution, and Elimination of the Brevetoxin PbTx-3 in Rats

12. PERSONAL AUTHOR(S)

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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Interim	FROM _____ TO _____	19 Oct 89	29

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	brevetoxin, distribution

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

CLEARANCE, DISTRIBUTION, AND ELIMINATION OF THE BREVETOXIN PbTx-3 IN RATS

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RUNNING TITLE: Brevetoxin Distribution and Elimination

M.A. POLI, C.B. TEMPLETON, W.L. THOMPSON and J.F. HEWETSON. Clearance, distribution, and elimination of the brevetoxin PbTx-3 in rats. Toxicon __, ___-___, 19__. - After intravenous administration, [^{3}H]PbTx-3 was rapidly cleared from the blood; less than 10% remained after 1 min. Within 30 min, radiolabel distributed to skeletal muscle (69.5%), liver (18.0%), and intestinal tract (8.0%). Over 24 hr, radiolabel decreased in muscle, remained constant in liver, and increased in the intestinal tract and feces. Elimination occurred via feces (75.1%) and urine (14.4%), with 9.0% remaining in the carcass after 6 days. This distribution and elimination profile suggested that liver was the major organ of metabolism and that biliary excretion was an important route of elimination. Thin-layer chromatography confirmed the presence of brevetoxin metabolites in fecal extracts. Skeletal muscle does not appear to be a site of metabolism, but a storage compartment, from which toxin is slowly released prior to clearance by the liver. These studies are the first demonstration of *in vivo* brevetoxin metabolism in mammals.

INTRODUCTION

Brevetoxins are cyclic polyether neurotoxins produced by the marine dinoflagellate Ptychodiscus brevis. Blooms of this organism, known as red tides, have long been reported along the gulf coast of the United States and recently along the Atlantic coast as far north as North Carolina (TESTER et al., 1988). To date, nine potent neurotoxins have been extracted and purified from P. brevis cell cultures (LIN et al., 1981; CHOU and SHIMIZU, 1982; GOLIK et al., 1982; BADEN, 1983; CHOU et al, 1985; WHITEFLEET- SMITH et al., 1986; BADEN, 1989). Each is a derivative of one of the two cyclic polyether backbones illustrated in Figure 1. Structural correlation of toxins from these different laboratories has revealed several redundancies in nomenclature that have been a source of confusion in the literature. The system of POLI et al., (1986), in which each toxin is preceded by the prefix "PbTx", is used in this report. Several excellent review articles on the structure and chemistry of the brevetoxins have appeared in the recent literature (ANDERSON et al., 1986; BADEN, 1989).

The pathophysiology of these compounds results from direct

effects on voltage-sensitive sodium channels in excitable cells (CATTERALL and RISK, 1981; HUANG et al., 1984; HUANG and WU, 1989). Electrophysiological studies (HUANG, et al., 1984, SHERIDAN and ADLER, 1989) have shown that exposure of neuronal cells to PbTx-3 causes depolarization, depression of the action potential and, eventually, complete block of axonal excitability. Activation voltage is shifted in the negative direction, leading to stimulation of a significant fraction of channels at resting membrane potentials. In mammalian cells, there appears to be no effect on the inactivation process (SHERIDAN and ADLER, 1989).

The toxins PbTx-2 and PbTx-3 have been shown to bind to a specific receptor site (site 5) that is distinct from other known neurotoxin receptor sites on the voltage-dependent sodium channel in rat brain synaptosomes (POLI, et al., 1986). Other brevetoxins have been demonstrated to displace radiolabeled PbTx-3 from this receptor site (BADEN, 1989).

Although recent advances in our knowledge of the site and mechanism of action of the brevetoxins have been great, there has been little or no information available regarding the metabolism and excretion of these compounds in vivo. This information is important for the screening of biological samples, and may reveal keys for designing rational approaches to the treatment of brevetoxin intoxication. A preliminary report of these results will appear as part of a conference proceedings (POLI, et al., 1989).

MATERIALS AND METHODS

Radiolabeled PbTx-3

[^{3}H]PbTx-3 (6.0-9.4 Ci/mmole) was prepared by Dr. D.G. Baden (University of Miami, Miami, FL) by the reduction of PbTx-2 with [^{3}H]sodium borohydride, as previously reported (POLI et al., 1986). Purity was greater than 99%, as indicated by a single UV-absorbing peak on HPLC analysis. A stock solution of 100 µg/ml in ethanol was kept in a freezer at -10°C and dilutions made immediately prior to use.

Plasma clearance

Male rats (Sprague-Dawley, 300-400 g, Charles River Laboratories, Wilmington, MA) were administered 1.5 µCi (9.4 Ci/mmole, 0.14 µg) [^{3}H]PbTx-3 via a catheter into the cranial vena cava. The toxin was given as a bolus injection in 200 µl phosphate-buffered saline, pH 7.4, containing 0.01% emulsifier (Emulphor EL-620, GAF Corp, New York, NY). Beginning immediately after toxin administration and continuing for 2 min, blood samples (three drops, 37 µl) were collected continuously from an aortic catheter directly into scintillation vials. These

samples were decolorized with 100 µl hydrogen peroxide for 2 hr at room temperature, acidified with 25 µl 2N HCl, and radioactivity measured in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Elimination

Male Sprague-Dawley rats (180-200 g) were administered an intravenous bolus dose of 19 µCi (9.4 Ci/mmole, 1.8 µg) [^{3}H]PbTx-3 into the penile vein in 0.5 ml phosphate-buffered saline containing 0.01% emulsifier. The animals were placed in glass metabolic cages and allowed food and water ad libitum. After 12 hr and every 24 hr for 6 days, urine and feces were collected and frozen. Feces samples were thawed, homogenized with distilled water in a total volume of 40 ml, and triplicate 0.2 ml aliquots oxidized with a Harvey OX300 Biological Oxidizer (R.J. Harvey Instrument Co., Hillsdale, NJ). Aliquots of urine (0.5 ml) were oxidized directly. The radioactivity in each sample was measured as above. To determine the amount of administered radiolabel remaining, the animals were killed after 6 days, and the carcasses dissolved in 750 ml of 2N sodium hydroxide at 85°C for 48 hr. Aliquots (0.25 ml) were acidified with 0.75 ml 2N HCL and counted as described above.

Organ distribution of [^{3}H]PbTx-3

Five groups of three male Sprague-Dawley rats (250-330 g) were

administered an intravenous bolus dose of 2.0 µCi (9.4 Ci/mmole, 0.19 µg) [^{3}H]PbTx-3 into the penile vein as described above. At intervals of 30 min, 3 hr, 6 hr, 12 hr and 24 hr, one group was killed; and hearts, kidneys, testes, lungs, stomachs, intestines, livers, and samples of skeletal muscle and fat were removed, weighed, and quickly frozen in liquid nitrogen. Tissue samples and small organs were analyzed for radioactivity by direct oxidation in the biological oxidizer as described above. Stomachs, intestines, and carcasses were dissolved in 2N NaOH for 96 hr at 85°C and aliquots were removed and oxidized as above. Radiolabel remaining in the carcass was determined by subtracting counts associated with skeletal muscle from the total carcass counts. For this calculation, skeletal muscle was assumed to constitute 40% of the total body weight (Charles River Breeding Labs, personal communication). Urine and feces were collected at 12 and 24 hr, and assayed as described above.

Thin-layer chromatography of fecal samples

Feces (3.39 g) collected at 48 hr was homogenized with distilled water with a Brinkmann tissue homogenizer (Brinkmann Instruments, Inc., Westbury, NY) in a total volume of 40 ml. This homogenate was stirred for 15 min at room temperature with two volumes of HPLC-grade acetone and filtered through Whatman #1 filter paper (Whatman Ltd., Maidstone, England). The filter cake was extracted twice more and the filtrates, containing 90-95% of

the total radioactivity, were combined and evaporated to dryness under vacuum. The residue was dissolved in 50 ml 90% methanol and liquid-liquid partitioned three times with 50 ml light petroleum ether. The ether fraction was back-extracted with 50 ml 90% methanol and this was added to the original methanol fraction. The ether fraction, which contained negligible radioactivity, was discarded. The 90% methanol fraction was evaporated to dryness under vacuum and the residue dissolved in 50 ml chloroform. The remaining chloroform-insoluble material was dissolved in 50 ml distilled water and extracted once with 25 ml fresh chloroform. This chloroform wash was added to the original chloroform fraction and evaporated to dryness under vacuum. The residue was then re-dissolved in 25 ml fresh chloroform. This fraction contained 85% of the extracted radiolabel with 15% remaining in the water fraction.

A 1-ml aliquot of the chloroform extract was streaked onto a 10 x 20 cm high-performance silica thin-layer chromatography (TLC) plate (Whatman High Performance Diamond System, HP-KF, 200 micron layer, Whatman Chemical Separations, Inc., Clifton, NJ). The plate was developed in a mobile phase of chloroform/ethyl acetate/95% ethanol (50/25/25), dried and developed again in the same solvent system in a ratio of 80/10/10. After drying, the plate was scanned for peaks of radioactivity with a Bioscan BID200 imaging scanner (Bioscan, Inc., Washington D.C.).

HPLC analysis of skeletal muscle extracts

Two male Sprague-Dawley rats (454-470 g) were infused with 25 ug/kg PbTx-3 containing 0.7 µg [^{3}H]PbTx-3 (9.4 Ci/mmole) as described for the plasma clearance experiments except that a 1-hr infusion was used rather than a bolus dose. This extended infusion time prevented the transient cardiac pathophysiology associated with a bolus injection of this dose of toxin. After 24 hr, the rats were killed and skeletal muscle was collected and frozen.

Frozen muscle tissue (37 g) was thawed and homogenized with distilled water in 40 ml total volume. This homogenate was extracted as described above except that, after partitioning with light petroleum ether, the 90% methanol fraction was evaporated and the residue taken up in 3 ml HPLC grade methanol. Samples of this extract were analyzed for [^{3}H]PbTx-3 and/or metabolites by HPLC with a reverse-phase C_{18} column, 250 x 4 mm, 5 µm particle size (BioRad Laboratories, Richmond, CA) and a nonlinear methanol/water gradient. The elution was begun at 40% methanol and this ratio held constant for 5 min, at which time the methanol was increased to 85% over 10 min and maintained at that level for an additional 15 min. Finally, the methanol concentration was decreased to 40% over 5 min. Flow rate for the mobile phase was maintained at 0.8 ml/min. Fractions from the column were collected with an LKB 2212 Helirac fraction collector (LKB-Producter AB, Bromma, SWE) and the radioactivity measured as

described above.

Data analysis

Data were analyzed by the Student's t-test to compare differences between means with significance set at $P<0.01$.

RESULTS

After intravenous administration, [^{3}H]PbTx-3 was rapidly cleared from the circulation; less than 10% remained after 1 min (Fig. 2). In the first 30 min after circulatory clearance, the radiolabel distributed to skeletal muscle (69.5%), liver (18.0%), and intestinal tract (8.0%) (Table 1). Over the course of 24 hr, radiolabel decreased to 18.9% in muscle, increased to 25.4% in the intestinal tract, and remained nearly constant in liver (11.7%). A small amount (1.6%) was detected in the stomach at this time. At all time points, only a small percentage of radiolabel was detected in the heart, kidney, testes, and lung. Previous experiments (data not shown) indicated negligible radiolabel (<0.05%) distributed to the brain and spleen. Radioactivity was detectable in the feces (6.7%) and urine (5.6%) after 12 hr, and increased to 21.2% and 8.2%, respectively, after

24 hr. At all time points greater than 30 min, a significant percentage of radiolabel (7.9-17.2%) remained in the carcasses.

Elimination occurred primarily through feces, although a small amount was detected in urine (Fig. 3). After 48 hr, 65% of the administered radiolabel had been eliminated via feces while only 10% was eliminated in the urine. Elimination was essentially complete by day 3. After 6 days, 75.1% of the radiolabel had been eliminated in the feces, 14.4% in the urine, and 9.0% was residual in the rats.

Thin-layer chromatographic analysis of fecal extracts (Fig. 4) indicated several peaks of excreted radiolabel that differed from the parent toxin. HPLC analysis of skeletal muscle extracts, however, indicated only the presence of parent toxin (Fig. 5). Samples of body fat collected at 30 min indicated radiolabel was present at 5,000-7,000 dis/min/g. However, the difficulty in determining the amount of total body fat in each animal precluded accurate quantification of this pool, and therefore fat samples from other time points were not analyzed.

DISCUSSION

Radiolabel distribution and elimination profiles described

here suggest that liver was the major organ of metabolism for PbTx-3, and that biliary excretion was an important route of elimination. Over the course of 24 hr after toxin administration, the proportion of radiolabel remained constant in liver as toxin was processed, yet increased in the intestinal tract and in feces as radiolabel was excreted via the bile. Thin-layer chromatography of lipid extracts of feces demonstrated several peaks of excreted radiolabel that differed from the parent toxin. This suggests that PbTx-3 is metabolized *in vivo* prior to elimination. While metabolism by gut flora cannot be ruled out as a source of fecal metabolites, other experiments with isolated perfused rat livers (PACE, *et al.*, 1987) and isolated rat hepatic cells (POLI *et al.*, 1989) exposed to [^{3}H]PbTx-3 have demonstrated time-dependent generation of radiolabeled brevetoxin metabolites and biliary elimination of these metabolites. Metabolism by other tissues also cannot be ruled out; but neither heart, kidney, testes, nor lung contained appreciable radiolabel at any time point, and the small amount of radiolabel eliminated in the urine may be explained by renal clearance of hepatic metabolites.

Skeletal muscle contained a significant proportion of the total administered radiolabel at all time points, although the concentration was always low on a per-gram basis. This probably resulted from the large proportion of the total body weight attributable to the muscle mass, and the relatively high

solubility of PbTx-3 in membrane lipids. We believe that the skeletal muscle is not a site of metabolism, but a storage compartment, from which free toxin is slowly released into the circulation and cleared by the liver prior to metabolism and excretion into the intestinal tract via the bile. This view is supported by HPLC analysis of radiolabel extracted from skeletal muscle, in which only parent toxin was present.

The radiolabel remaining in the carcass after 24 hr (11.7%) was not significantly different than that after 6 days (9.0%). This included label in all other tissues as well as that dissolved in body fat. As body fat is very poorly vascularized, and PbTx-3 is highly lipophilic, radiolabel may clear this compartment slowly, perhaps requiring weeks for complete depuration.

These experiments constitute the first demonstration of *in vivo* brevetoxin metabolism in mammals. So far, structural determinations have been hindered by a shortage of purified metabolites. Isolation and structural analysis of the major metabolites should yield important information on biochemical processing of PbTx-3 *in vivo*, and therefore may suggest viable methods of treatment. Further, if biological samples such as serum, urine, or feces are to be assayed to determine brevetoxin exposure, then targeting the appropriate structures is crucial. Toward these ends, further isolation, purification and direct comparison of both *in vivo* and *in vitro* metabolites is in

progress.

Acknowledgements

All procedures performed in this study conform to the "Guide to the Care and Use of Laboratory Animals" published by the National Institutes of Health, Bethesda, Maryland. All research facilities are accredited by the American Association for Accreditation of Laboratory Animal Care.

The opinions of the authors in no way reflect the opinions of the Department of the Army or the Department of Defense.

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FIG. 1. STRUCTURES OF THE BREVETOXINS

(A) PbTx-2 and derivatives:

PbTx-2: R_1=H, R_2=$CH_2C(=CH_2)CHO$

PbTx-3: R_1=H, R_2=$CH_2C(=CH_2)CH_2OH$

PbTx-5: R_1=Ac R_2=$CH_2C(=CH_2)CHO$

PbTx-6: R_1=H R_2=$CH_2C(=CH_2)CHO$ (27,28 epoxide)

PbTx-8: R_1=H R_2=CH_2COCH_2Cl

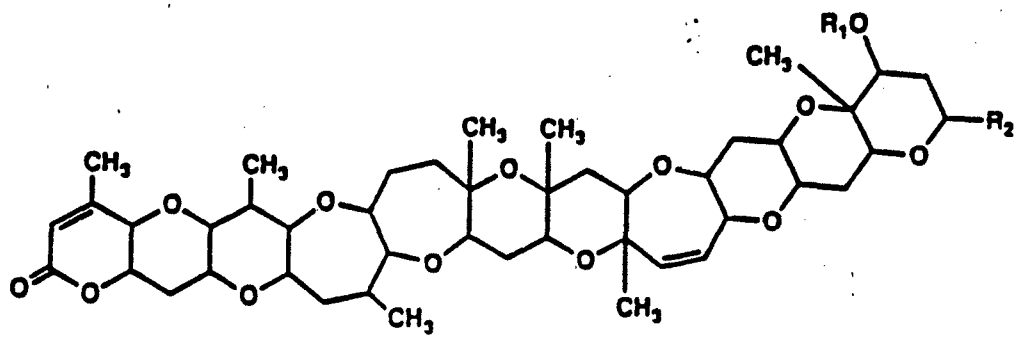
PbTx-9: R_1=H R_2=$CH_2CH(CH_3)CH_2OH$

(B) PbTx-1 and derivatives:

PbTx-1: R=$CH_2C(CH_2)CHO$

PbTx-7: R=$CH_2C(CH_2)CH_2OH$

PbTx-10: R=$CH_2CH(CH_3)CH_2OH$



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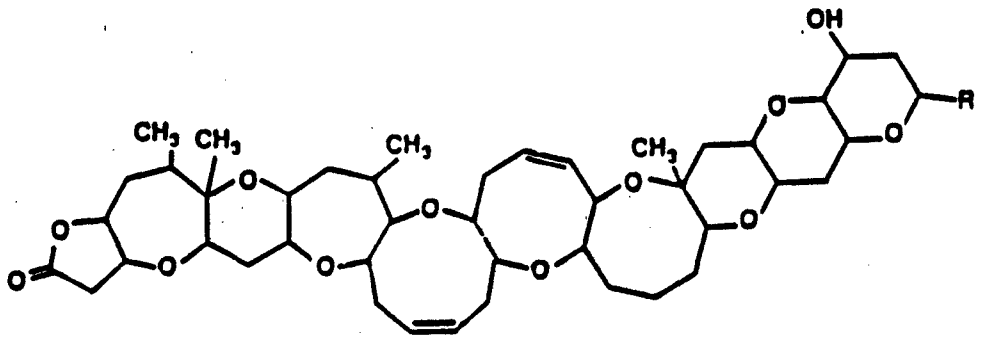


B

FIG. 2. CIRCULATORY CLEARANCE OF RADIOLABEL IN RATS AFTER INTRAVENOUS ADMINISTRATION OF [^{3}H]PbTx-3

Two rats were administered 1.5 µCi (0.14 µg) [^{3}H]PbTx-3 as a bolus injection via catheter into the cranial vena cava. Beginning immediately after toxin injection, blood samples (three drops, 37 µl) were collected continuously from an aortic catheter directly into scintillation vials and assayed for radioactivity. Each line represents data from a single animal.

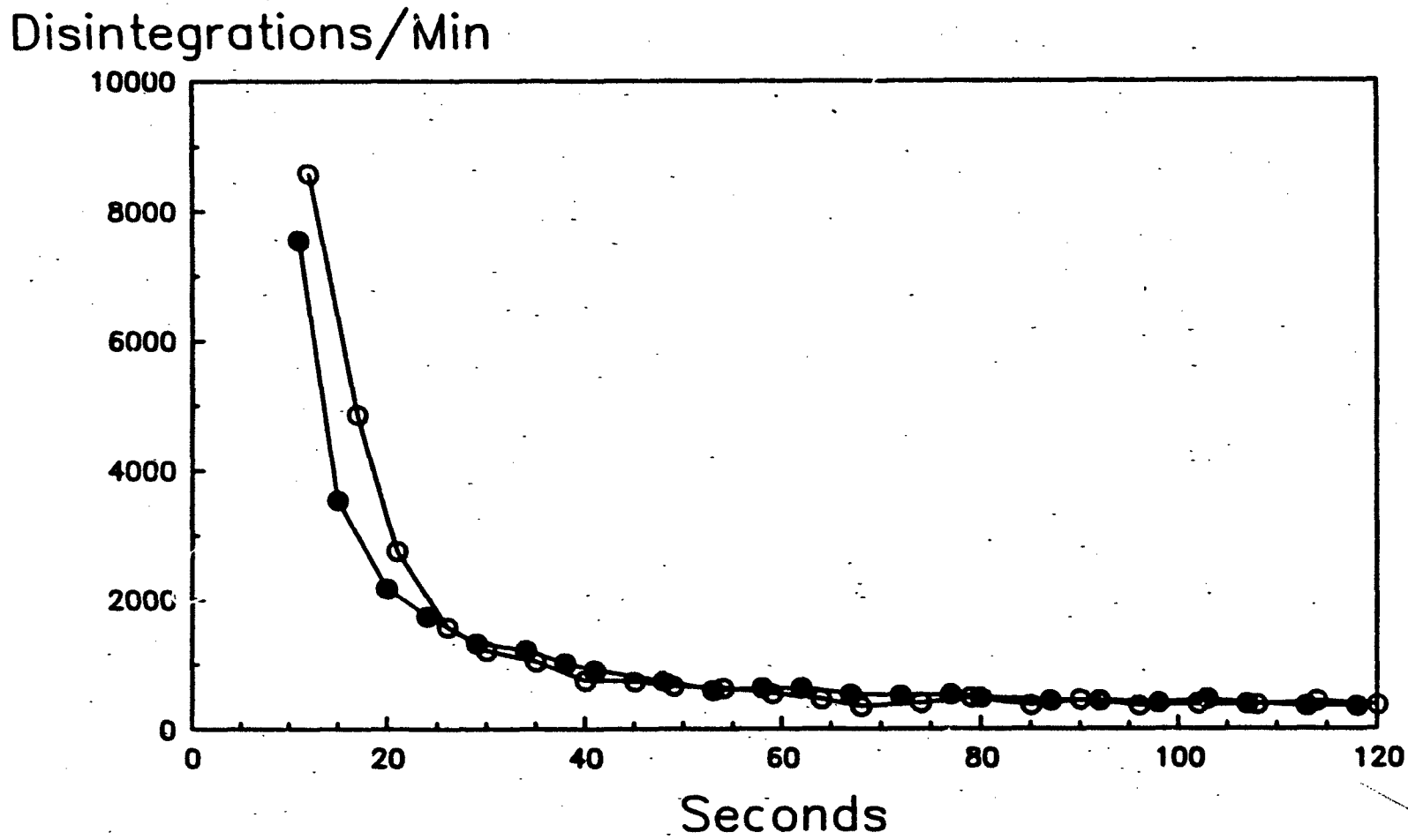

Disintegrations/Min
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
120
Seconds

TABLE 1. DISTRIBUTION OF RADIOLABEL IN RAT TISSUES AFTER INTRAVENOUS ADMINISTRATION OF [^{3}H]PbTx-3

TISSUE	PERCENT TOTAL RECOVERED DOSE				
	30 min	3 hr	6 hr	12 hr	24 hr
HEART	1.6% (0.23)	1.0% (0.06)	1.1% (0.06)	0.4% (0.02)	0.3% (0.03)
KIDNEY	1.4% (0.01)	1.1% (0.06)	0.9% (0.01)	0.5% (0.04)	0.5% (0.03)
TESTES	0.4% (0.03)	0.5% (0.01)	0.3% (0.01)	0.4% (0.02)	0.3% (0.40)
LUNG	0.4% (0.03)	0.5% (0.05)	0.4% (0.03)	0.2% (0.02)	0.3% (0.03)
STOMACH*	0.4% (0.01)	0.6% (0.1)	0.7% (0.03)	0.3% (0.06)	1.6% (0.3)
MUSCLE**	69.5% (2.9)	56.0% (1.1)	39.2% (1.0)	23.6% (0.5)	18.9% (2.4)
LIVER	18.0% (1.6)	16.7% (0.1)	18.6% (1.4)	11.8% (0.5)	11.7% (0.4)
INTESTINES*	8.0% (0.3)	15.7% (0.4)	21.5% (1.6)	37.0% (1.3)	25.4% (1.2)
FECES	NC	NC	NC	6.7% (2.6)	21.2% (2.3)
URINE	NC	NC	NC	5.6% (0.3)	8.2% (4.7)
CARCASS†	0.3% (2.0)	7.9% (1.8)	17.2% (0.7)	13.5% (0.2)	11.7% (1.5)

Values represent means (SEM); n=3
* Stomach and intestines were analyzed with contents
**Skeletal muscle was assumed to constitute 40% of total weight
†Radiolabel associated with skeletal muscle has been subtracted
NC: not collected

FIG. 3. ELIMINATION OF RADIOLABEL AFTER INTRAVENOUS ADMINISTRATION OF [^{3}H]PbTx-3 TO RATS

Rats were administered 19 µCi (1.8 µg) [^{3}H]PLTx-3 as a bolus intravenous dose and housed in metabolic cages for 6 days. Urine (▲) and feces (●) were collected and assayed for radioactivity at the indicated time intervals. Each point represents the mean (±SD) of three animals.

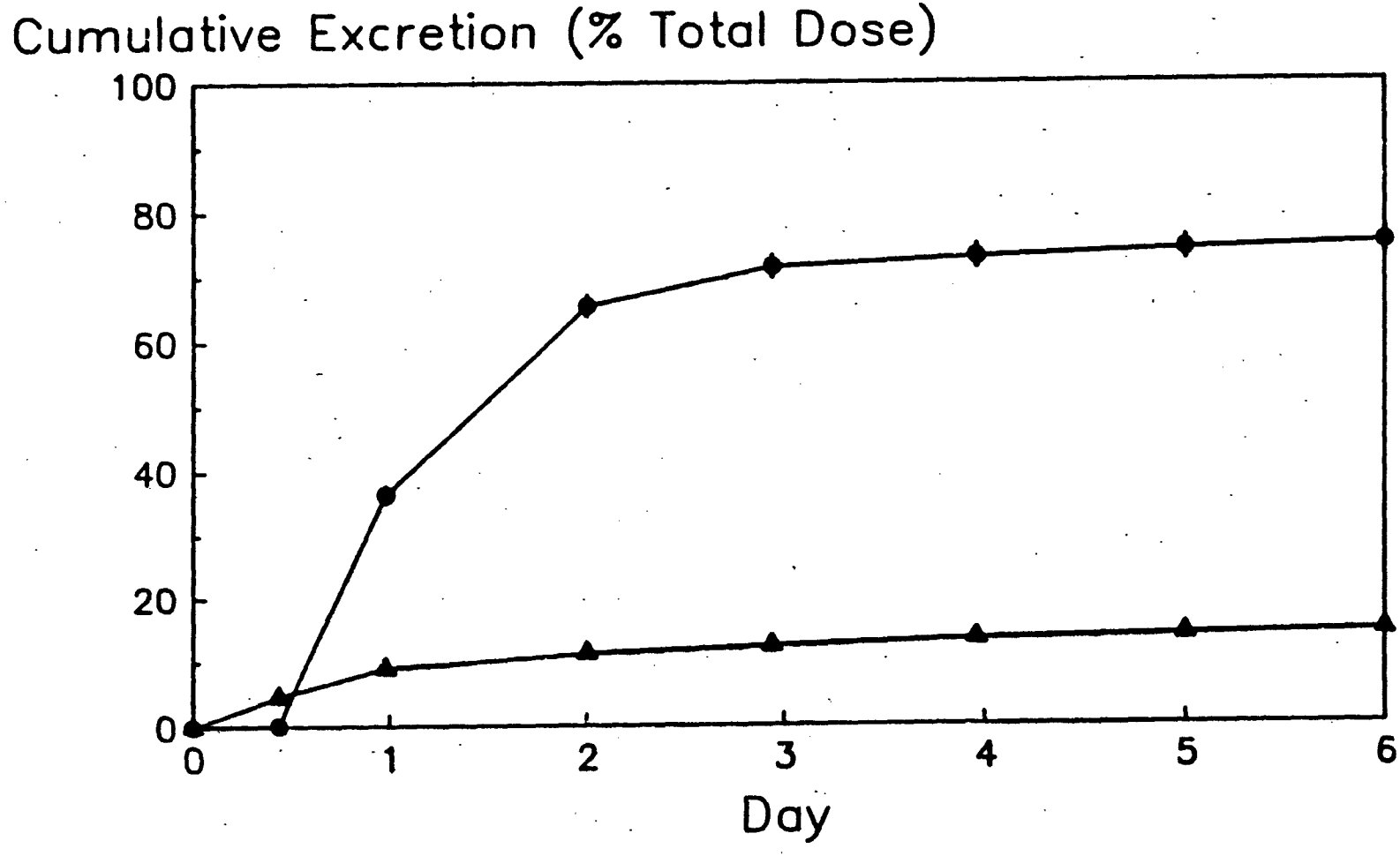

Cumulative Excretion (% Total Dose)
100
80
60
40
20
0
0
1
2
3
4
5
6
Day

FIG. 4. THIN-LAYER CHROMATOGRAPHY OF RAT FECAL EXTRACTS AFTER INTRAVENOUS ADMINISTRATION OF [^{3}H]PbTx-3

Rat feces collected 48 hr after intravenous administration of [^{3}H]PbTx-3 was lipid-extracted as described in Materials and Methods. An aliquot of this extract was chromatographed on a silica gel 200 µm high performance TLC plate by a two-step development in a mobile phase of chloroform/ethyl acetate/95% ethanol (50/25/25 followed by 80/10/10). The plate was scanned for radioactivity by a Bioscan BID200 imaging scanner. Several radioactive peaks resolved which were distinct from the parent toxin, suggesting <u>in vivo</u> metabolism of [^{3}H]PbTx-3. In a separate run, [^{3}H]PbTx-3 standard migrated as a single peak with R_f = 0.74.

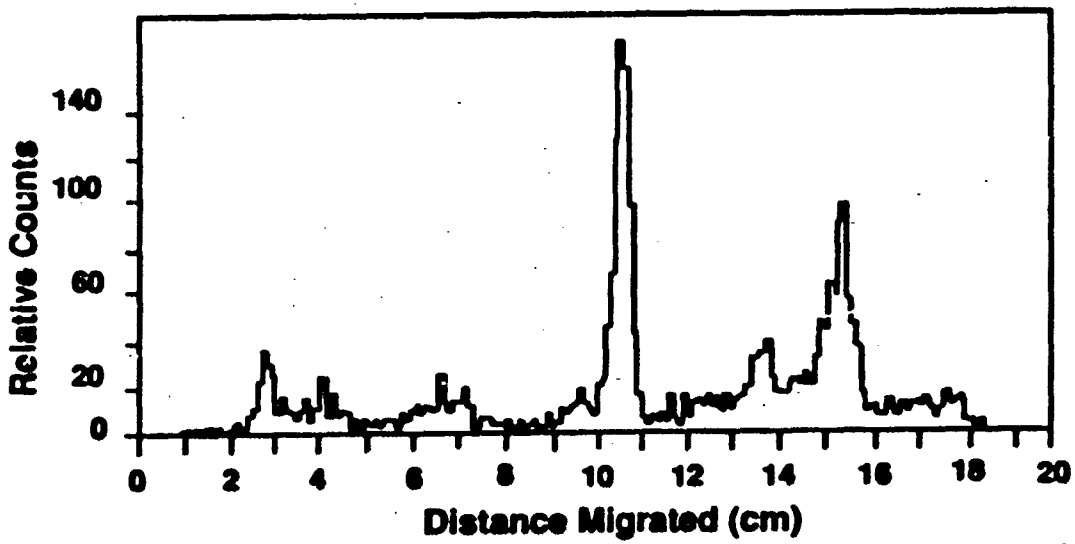
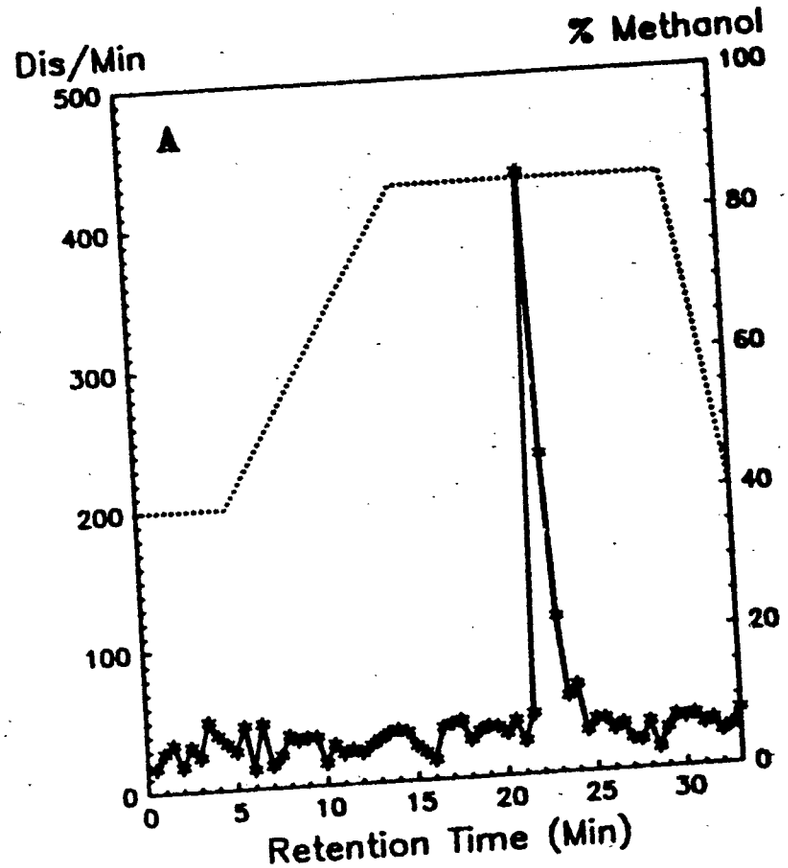
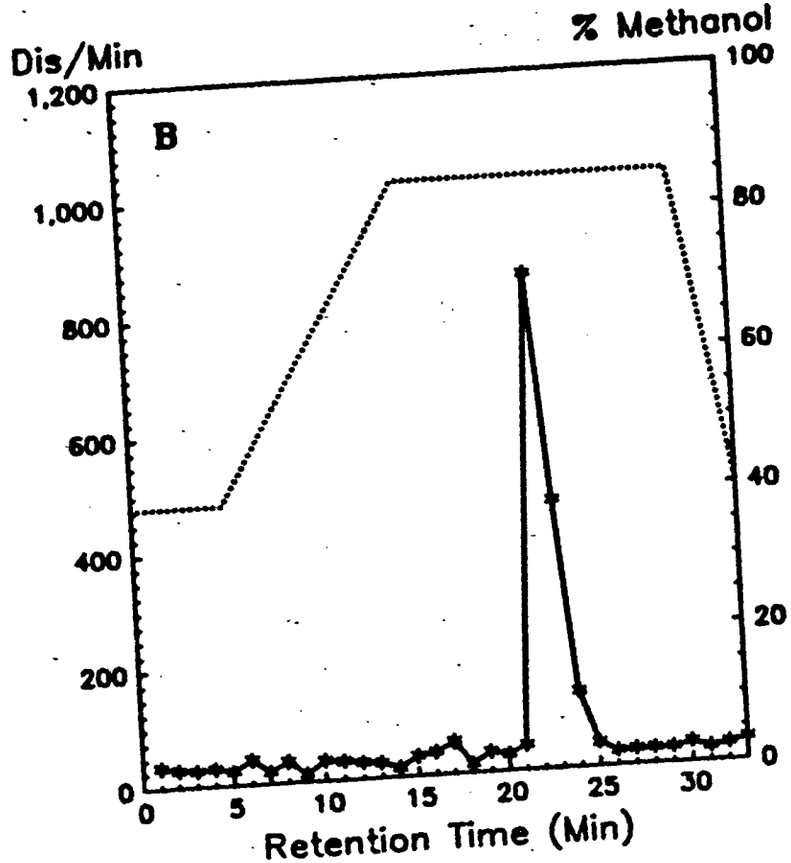

Relative Counts
140
100
60
20
0
0
2
4
6
8
10
12
14
16
18
20
Distance Migrated (cm)

FIG. 5. HPLC ANALYSIS OF RADIOLABEL EXTRACTED FROM RAT SKELETAL MUSCLE AFTER INTRAVENOUS ADMINISTRATION OF [^{3}H]PbTx-3

Rat skeletal muscle collected 24 hr after a 1-hr intravenous infusion of [^{3}H]PbTx-3 was extracted as described in Materials and Methods. The extract was analyzed by reverse phase HPLC on a 250 x 4 mm C_{18} analytical column using a nonlinear methanol/water gradient as the mobile phase. Fractions were collected and analyzed for radioactivity by liquid scintillation counting. The flow rate was maintained at 0.8 ml/min. (A) Skeletal muscle extract, (B) [^{3}H]PbTx-3 standard.


Dis/Min
% Methanol
A
500
400
300
200
100
0
100
80
60
40
20
0
0
5
10
15
20
25
30
Retention Time (Min)


Dis/Min
% Methanol
B
1,200
1,000
800
600
400
200
0
100
80
60
40
20
0
0
5
10
15
20
25
30
Retention Time (Min)